

Exhibit G

Rapid Communication

Alternative Splicing of Human VCAM-1 in Activated Vascular Endothelium

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Vascular cell adhesion molecule 1 (VCAM-1)/inducible cell adhesion molecule 110 is a mononuclear leukocyte-selective adhesion molecule, expressed on vascular endothelium following activation by certain cytokines or endotoxin. This inducible transmembrane protein and member of the immunoglobulin gene superfamily was previously reported to contain six immunoglobulinlike domains. Using the polymerase chain reaction, a VCAM-1 cDNA was obtained from mRNA of interleukin-1 (IL-1)-treated cultured human umbilical vein endothelial cells (HUVEC). The cDNA clone contained an additional 276 base-pair (bp) domain, located between domains 3 and 4. This new domain is most homologous to the existing N-terminal domain (domain 1). The internal 276-bp region is encoded by a single exon of the human VCAM-1 gene, indicating that the two forms of mRNA arise by alternative splicing. Both forms of VCAM-1 mRNA were detected by polymerase chain reaction in IL-1-stimulated HUVEC, although the seven-domain form appeared predominant. On the surface of HUVEC only a 110-kd polypeptide, consistent with the seven-immunoglobulinlike domain form of VCAM-1, was detectable by immunoprecipitation. Alternative splicing of the VCAM-1 gene in cytokine-activated endothelium may generate functionally distinct cell-surface adhesion molecules. (*Am J Pathol* 1991, 138:815-820)

The activation of endothelium by cytokines or endotoxin is a pathophysiologic response which alters the homeostatic functions of these vascular lining cells. A prominent

feature is an alteration of endothelial surface adhesive properties by induction of leukocyte adhesion molecule expression (reviewed in Cybulsky and Gimbrone¹). These molecules contribute to the hyperadhesive surface change observed in activated cultured endothelium, and have been identified *in vivo* in various pathologic conditions.¹ To date three inducible leukocyte adhesion molecules have been identified in endothelium and molecularly cloned, each with a unique expression profile and function. These include endothelial-leukocyte adhesion molecule 1 (ELAM-1), a member of the selectin family,² intercellular adhesion molecule 110 (ICAM-1),^{3,4} and vascular cell adhesion molecule 1 (VCAM-1)/inducible cell adhesion molecule 1 (INCAM-1).^{5,6} The latter two members of the immunoglobulin gene superfamily.⁷

VCAM-1/INCAM-110 was identified independently by two different strategies. One used a monoclonal antibody produced to tumor necrosis factor (TNF)-activated human umbilical vein endothelial cells (HUVEC) that identified an inducible 110-kd surface protein, designated INCAM-110, which supported the adhesion of mononuclear leukocytes and certain tumor cells.^{6,8} The other used a eukaryotic expression cloning strategy and identified a cDNA clone from an interleukin-1 (IL-1)-stimulated HUVEC, designated VCAM-1, which supported the adhesion of myelomonocytic and lymphocytic leukocyte cell lines.⁵ This cDNA encodes a transmembrane protein with six immunoglobulinlike domains.⁵ Subsequently the antibody to INCAM-110 was found to bind specifically to SV40-transformed African green monkey kidney (COS) cells transfected with a polymerase chain reaction (PCR)-produced VCAM-1 cDNA clone.⁹ The leukocyte ligand for VCAM-1 is the integrin $\alpha^4\beta_1$ (VLA-4,

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CD49d/CD29), a heterodimeric molecule present on monocytes and lymphocytes, but not neutrophils; consequently VCAM-1 selectively supports mononuclear leukocyte adhesion.⁹ *In vivo* VCAM-1 expression is induced on vascular endothelium of postcapillary venules in pathologic conditions associated with inflammation.^{8,10} In the present report, we show that alternative splicing of the human VCAM-1 gene gives rise to a protein with seven immunoglobulinlike domains, which appears to be the predominant form of VCAM-1 in activated HUVEC.

Methods

Cloning of VCAM-1 from Human Umbilical Vein Endothelium Using the Polymerase Chain Reaction

Second culture HUVEC isolated from two to six umbilical cords² were treated with 10 U/ml of recombinant human IL-1 beta (Biogen, Boston, MA) for 6 hours at 37°C; cytoplasmic RNA was prepared by a detergent lysis protocol.¹¹ First strand cDNA was synthesized during a 1-hour incubation (42°C, final reaction volume of 40 μ l), using 1 μ g of RNA as template, 50 ng of an oligonucleotide primer complementary to the sequence found in the 3' untranslated region of VCAM-1 mRNA: GGGTCATATAGTCTTGTAGAACACAGAAATC, and 100 U of avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources, Tampa, FL). Two rounds of PCR were performed using nested primers, and 5 μ l of first-strand cDNA or first-round PCR products as templates.¹² Three hundred ng of primers were used for the first round of PCR and 150 ng for the second round. A primer to the 5' untranslated region of the mRNA: GAGCTGAATACCCCTCCAGGCACACACAGGTG and the same 3' primer used in cDNA synthesis were used for first-round PCR. The nested set of primers consisted of GGGTTTTGGAACCACTATTTTCTCATC and a sequence complementary to GTTTAACACTTGATGT-TCAAGGAAGAGAAAATAA. Both rounds of PCR were 26 cycles performed under the following conditions: 1 minute of denaturing at 94°C, 2 minutes of annealing at 50°C, and 6 minutes of extension at 72°C with a 40-minute extension time in the first cycle. Polymerase chain reaction products (10 μ l) were analyzed by standard agarose gel electrophoresis and Southern blotting.¹¹

The prominent 2.05-kb reaction product from a parallel set of six PCR reaction tubes was pooled and purified. The ends of the PCR product were repaired with the Klenow fragment of DNA polymerase I, phosphorylated with T4 kinase, and the fragment was subcloned into the HindIII site of the plasmid vector pBS (Stratagene, La Jolla, CA), according to standard procedures.¹¹

Cloning and Sequencing of the Human VCAM-1 Gene

A bacteriophage lambda library of human peripheral blood DNA in the vector EMBL3¹³ was plated, nitrocellulose filters prepared according to standard procedures,¹¹ and screened with a rabbit partial cDNA for VCAM-1 (Cybulsky et al, manuscript in preparation). The cDNA probe was labeled with Klenow fragment of DNA polymerase I in the presence of hexanucleotide primers and [α -³²P] dCTP.¹⁴ Filters were incubated with the radiolabeled probe in 6 \times SSC buffer, 0.5% sodium dodecyl sulfate (SDS) at 65°C, and washed with 0.5 \times SSC, 0.5% SDS at 65°C. Hybridizing bacteriophages were purified and amplified, bacteriophage DNA prepared, and restriction fragments containing the VCAM-1 gene ligated into the plasmid vector pBS.¹¹

Nucleotide sequences were determined by the dideoxynucleotide chain termination procedure with modified T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH) and [α -³⁵S]-dATP. Oligonucleotide primers were synthesized using an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA) and used without purification.

Immunoprecipitation of VCAM-1 from HUVEC

Total cell lysates in 1% Triton X-100 (Pierce, Rockford, IL) were prepared from surface-iodinated TNF-treated HUVEC (2000/ml, for 24 hours, Biogen), as previously described.¹⁵ Lysates (approximately 5 \times 10⁵ cells in 100 μ l) were precleared with nonbinding murine antibody, then incubated with 100 μ l of primary monoclonal antibody (4 hours, 4°C), followed by goat antibody to murine IgG coupled to Sepharose-4B (Organon Teknika, West Chester, PA for 2 hours at 4°C). Sepharose beads were pretreated with unlabeled lysate to diminish nonspecific adherence of labeled proteins. Antigens that were bound specifically to Sepharose beads were extensively washed and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 5% to 12% linear gradient gels.¹⁵ Monoclonal antibody E1/6 was provided by M. Bevilacqua, and immunostained COS cells transfected with our VCAM-1 cDNA (data not shown).

Results

Interleukin-1-treated HUVECs express the VCAM-1 gene.⁵ A partial cDNA corresponding to the extracellular region of VCAM-1 was generated by PCR. This cDNA was 2.05 kb in length, and about 0.25 kb longer than would be predicted from the existing human VCAM-1 sequence. Based on sequencing, the coding region of this amplified cDNA was identical to the previously re-

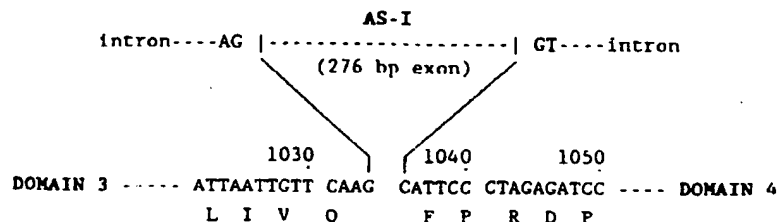
ported VCAM-1 HUVEC cDNA clone⁵ from amino acid residue 1 to 309, at which point the sequences diverge. At the nucleotide level, the sequences were identical to bp 1034 of the published sequence,² and after an insertion of 276 bp (Figure 1), the two sequences resumed identity. The differences in the transcript structure lengthens the predicted protein by 92 residues between the third and fourth immunoglobulin domains. Recently a VCAM-1 cDNA containing the same 276-bp insertion was identified by Polte et al.¹⁶

The amino acid sequence of this additional region, designated AS-I, was homologous to other members of the immunoglobulin gene superfamily. Members of this superfamily contain regions consisting of a disulfide-bridged loop that has a number of antiparallel beta-pleated strands arranged in two sheets. Three types of immunoglobulin homology regions (V, C and C2, or H) have been defined, each with a typical length and consensus of amino acid residues at certain positions relative

to the two cysteine residues that form the bridge.⁷ AS-I is a domain of the C2 or H type, consistent with the other six extracellular domains of VCAM-1. Furthermore this sequence was 73% homologous with the N-terminal immunoglobulinlike domain (domain 1) (Figure 2). The AS-I domain also contained an additional potential N-linked glycosylation site.

To demonstrate that two VCAM-1 transcripts were derived by alternative mRNA splicing, the corresponding region of the human VCAM-1 gene was cloned from a human genomic library. A phage clone was obtained by screening a human genomic library with a rabbit partial cDNA. Exons, intron-exon boundaries, and portions of the introns of the VCAM-1 gene were identified and sequenced (manuscript in preparation). The AS-I domain corresponded to a single exon located between exons containing domains 3 and 4. Splice donor and acceptor sequences flanking the AS-I exon conform to consensus.¹⁷ The nucleotide sequence of the AS-I exon

Figure 1. Nucleotide, deduced amino acid sequence, and location of alternatively spliced exon AS-I with respect to VCAM-1 cDNA. The potential N-linked glycosylation site is underlined.



AS-I DOMAIN:

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50
AGAAAGCATT TACIGTTGAG ATCTCCCTG GACCCCGGAT TGCTGCTCAG
E K P F T V E I S P G P R I A A Q

100
ATTGGAGACT CAGTCATGTT GACATGTAGT GTCATGGGCT GTGAATCCCC
I G D S V M L T C S V M G C E S P

150
ATCTTTCTCC TGGAGAAGCC AGATAGACAG CCCTCTGAGC GGAAGGTGA
S F S W R T Q I D S P L S G K V R

200
GGAGTGAGCG GACCAATTCG ACGGTGACCC TGAGCCCTGT GAGTTTGTAG
S E G T N S T L T L S P V S F E
-----
250
AACGAACACT CTTATCTGTG CACACTGACT TGTGGACATA AGAACTGGA
N E H S Y L C T V T C G H K K L E

276
AAAGGCAATC CAGGTGACGC TCTACT
K G I Q V E L Y T
  
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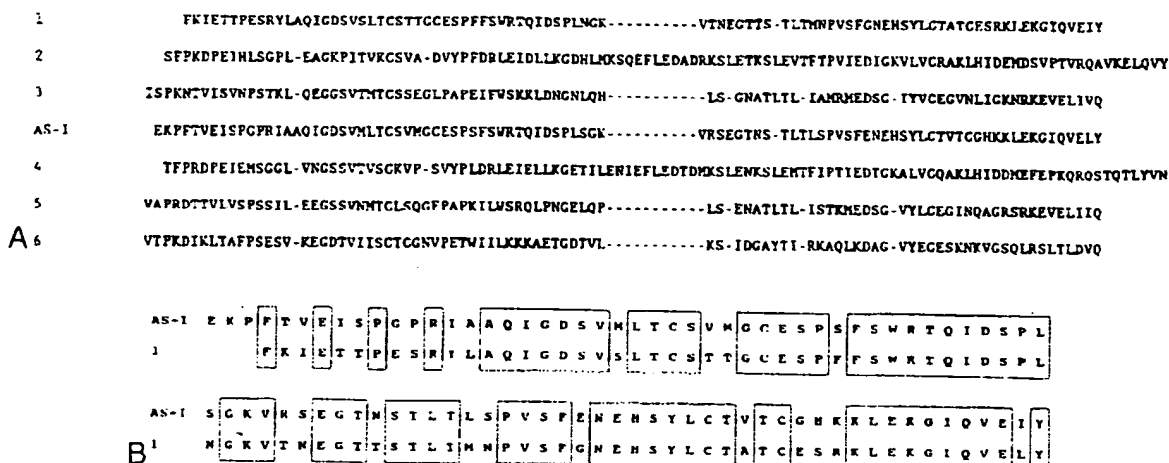


Figure 2. A: Extracellular structure of the seven immunoglobulinlike domain form of VCAM-1. The sequence was aligned to residues conserved in C2 or H type immunoglobulin regions. Cysteine residues forming disulfide bridges in each domain are indicated with arrowheads. B: Structural homology between AS-1 and domain 1. Sequences were aligned by inspection, and homologous regions boxed.

was identical to that of the cDNA sequence obtained by PCR amplification. Although the PCR that we performed was not quantitative, the 2.05-kb product generated by amplification of VCAM-1 extracellular domains (Figure 1) suggested that a seven-domain form was the predominant mRNA species. To confirm that IL-1-activated HUVEC express both forms of VCAM-1 mRNA, we again used nested PCR. The same primers and protocols were used for first-strand cDNA synthesis and first-round PCR; however the nested primers were selected to regions in

domains 3 and 5 (AATTATGTGTGTGAAGGAG and TTCTGTGAATATGACAT, respectively). By ethidium bromide staining and Southern blotting with oligonucleotide probes to regions of domain 3, AS-1, and 4, the predominant PCR product was 740 bp (Figure 3A), consistent with the seven-domain form. However a 464-bp product that did not hybridize the AS-1 probe also was identified, confirming that the six immunoglobulin domain form of VCAM-1 was expressed by HUVEC.

To determine which form of VCAM-1 protein was ex-

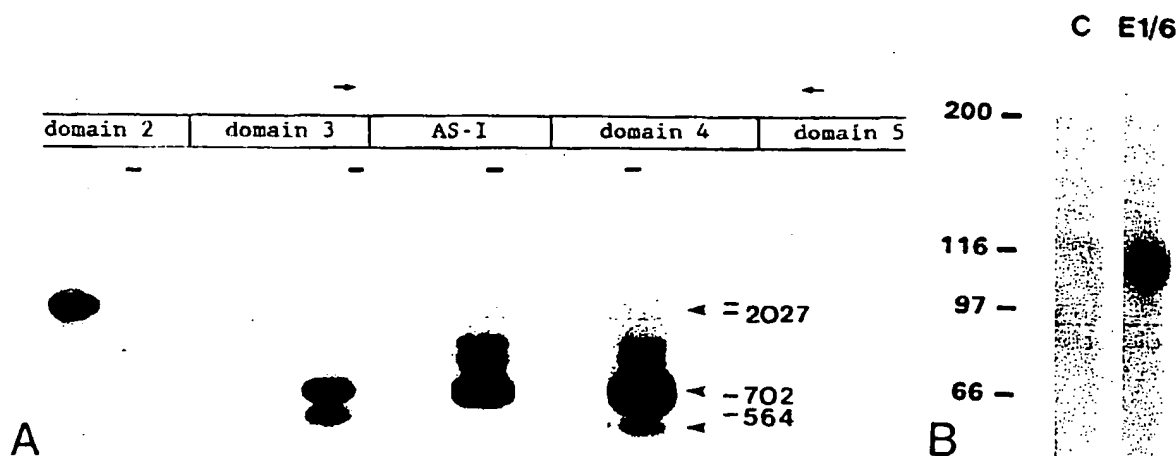


Figure 3. A: Identification of seven and six immunoglobulinlike domain forms of VCAM-1 mRNA in IL-1-activated endothelium. Polymerase chain reaction was performed with primers corresponding to regions of domains 3 and 5, as indicated by arrows. These primers should generate 740- and 464-bp products from VCAM-1 cDNA with and without the AS-1 domain, respectively. Southern blotting using oligonucleotide probes corresponding to the underlined regions of domains 3, AS-1, and 4 confirmed the presence of both species. As expected, the domain 3 and 4 probes hybridized to both 740- and 464-bp species, whereas the AS-1 probe hybridized only to the 740-bp product. A domain 2 probe, which was 5' of the region amplified by PCR, did not hybridize with either product (right lane). All the above probes hybridized to the 2.05-kb PCR product generated previously using primers spanning the seven extracellular immunoglobulinlike domains of VCAM-1 (results with domain 2 probe shown, left lane). Relevant hybridization is indicated by arrowheads. Hybridizing higher molecular weight bands probably represent PCR artifact or may be novel forms of VCAM-1. Molecular weight standards are bacteriophage lambda DNA digested with Hind III and Bst E II (right). B: Detection of the seven immunoglobulin domain VCAM-1 protein on the surface of activated endothelium. Immunoprecipitation with monoclonal antibody E1/6 identified a 110 kd polypeptide from surface-iodinated TNF-activated HUVEC lysates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions on 5% to 12% linear gradient gel (C, nonbinding IgG1 monoclonal antibody).

pressed on the surface of endothelium, surface iodination and immunoprecipitation was performed. A 110-kd polypeptide, consistent with the seven immunoglobulin domain form, was specifically immunoprecipitated by monoclonal antibody E1/6 directed to VCAM-1 (Figure 3B).

Discussion

Our data indicate that the six and seven immunoglobulin domain forms of VCAM-1 mRNA are derived by alternative splicing. In HUVEC activated with IL-1, the seven immunoglobulin domain form of VCAM-1 appeared predominant. We observed this splice pattern at 1 through 24 hours of IL-1 activation and noted that a different stimulus, endotoxin, induced in cultured rabbit endothelium predominantly an AS-I domain-containing form of VCAM-1 (data not shown). It is conceivable that other pathophysiologic stimuli, or expression in other cell types may have different splicing patterns. Tissue-specific alternative splicing has been described in the homologous neural cell adhesion molecule, N-CAM.^{18,19} This possibility remains to be investigated in nonvascular cells in which VCAM-1 expression is found, including dendritic cells in lymphoid tissues and skin, monocyte-derived cells in liver (Kupffer cells) and spleen, and some epithelial cells in the kidney.^{8,10}

Our observation that the seven immunoglobulin domain form of VCAM-1 mRNA appears predominant in activated HUVEC is consistent with expression of a 110-kd protein, determined by immunoprecipitation of biosynthetically labeled cells with monoclonal antibody E1/6.⁶ E1/6 blocked leukocyte adhesion to activated HUVEC,^{6,8} and immunoprecipitation of surface-iodinated HUVEC with E1/6 detected only the 110-kd polypeptide (Figure 3B). Together these data suggest that on the activated HUVEC surface, it is the seven-domain form that supports adhesion of mononuclear leukocytes. The six-domain VCAM-1 cDNA expressed on transfected COS cells also supports mononuclear leukocyte adhesion.^{5,9} In light of our demonstration of alternative splicing of the human VCAM-1 gene, the precise function of the protein products will require re-examination. For example, it is possible that the seven- and six-domain forms may have different affinities or even some difference in specificity for leukocytes or tumor cells.

Recently we have identified in the rabbit a mononuclear leukocyte adhesion molecule whose induction on arterial endothelium is localized to developing atherosclerotic lesions.¹⁵ This molecule is expressed as two distinct polypeptides, the NH₂-terminal amino acid sequence of each being highly homologous with the predicted sequence of VCAM-1.¹⁵ Alternative splicing may account for the two forms of this molecule, and in arterial

endothelium generate structures that have an important role in the pathogenesis of atherosclerosis.

Acknowledgments

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References

1. Cybulsky MI, Gimbrone MA Jr: Endothelial leukocyte adhesion molecules in acute inflammation and atherogenesis. In Simionescu N, Simionescu M, eds. *Endothelial Cell Dysfunction*. Plenum Publishing Corp., 1991 (In press)
2. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B: Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* 1989; 234:1160-1165
3. Simmons D, Makgoba MW, Seed B: ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. *Nature* 1988; 331:624-627
4. Staunton DE, Martin SD, Stratowa S, Dustin ML, Springer TA: Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell* 1988; 52:925-933
5. Osborn L, Hession C, Tizard R, Vassallo C, Luthowskyj S, Chi-Rosso G, Lobb R: Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds lymphocytes. *Cell* 1989; 59:1203-1211
6. Rice GE, Bevilacqua MP: An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science* 1989; 246:1303-1306
7. Hunkapiller T, Hood L: Diversity of the immunoglobulin gene superfamily. *Adv in Immunol* 1989; 44:1-63
8. Rice GE, Munro JM, Bevilacqua MP: Inducible cell adhesion molecule 110 is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism. *J Exp Med* 1990; 171:1369-1374
9. Ellices MJ, Osborn L, Takada Y, Crouse C, Luthowskyj S, Hemler ME, Lobb RR: VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4/fibronectin binding site. *Cell* 1990; 60:577-584
10. Rice GE, Munro JM, Corfess C, Bevilacqua MP: Vascular and nonvascular expression of INCAM-110: A target for mononuclear leukocyte adhesion in normal and inflamed human tissues. *Am J Pathol* 1991; 138:385-393
11. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor, NY, New York, Cold Spring Harbor Laboratory, 1989
12. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988; 239:487-491
13. Bonthron DT, Morton CC, Orkin SH, Collins T: Platelet-

- derived growth factor A chain: Gene structure, chromosomal location and basis for alternative splicing. *Proc Natl Acad Sci USA* 1988, 85:1492-1496
14. Feinberg AP, Vogelstein B: A technique for radiolabeling DNA endonuclease restriction fragments to high specific activity. *Anal Biochem* 1983, 132:6-13
15. Cybulsky MI, Gimbrone MA Jr: Expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 1991, 251:788-791
16. Poite T, Newman W, Gopal TV: Full length vascular cell adhesion molecule 1 (VCAM-1). *Nucleic Acids Res* 1990, 18:5901
17. Mount SM: A catalogue of splice junction sequences. *Nucleic Acids Res* 1982, 10:459-472
18. Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM: Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation and alternative splicing. *Science* 1987, 236:799-806
19. Dickson G, Gower HJ, Barton CH, Prentice HM, Elsom VL, Moore SE, Cox RD, Quinn C, Putt W, Walsh FS: Human muscle neural cell adhesion molecule (N-CAM): Identification of a muscle-specific sequence in the extracellular domain. *Cell* 1987, 50:1119-1130

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